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Focus

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Molecular inroads towards progress in HCC

Efforts to understand hepatocellular carcinoma (HCC) are at a fever pitch because of the epidemic incidence of the disease and the paradigm-shifting SHARP trial [1], which established this neoplasm as a tractable target for molecular therapies. As a result, identifying new molecular targets and improving the diagnosis of early disease are major points of attack, and two articles from this month's issue address these important topics.

The study by Tomimaru *et al.* reports detection of microRNA-21 (miR-21) as a biomarker of disease in hepatocellular carcinoma, which is similar to previous reports detecting circulating miRNAs as markers of HCC [2,3], including one also describing serum miR-21 [3]. The Tomimaru study, like the previous miR-21 report [3], notes elevated levels both in chronic hepatitis as well as HCC. The Tomimaru study suggests that elevated miR-21 is comparable to alpha fetoprotein (AFP) in distinguishing chronic hepatitis from HCC, and could improve the diagnostic power of AFP. Supporting the contention that elevated miR-21 is derived directly from HCC, plasma levels consistently decreased after hepatic resection for HCCs in 126 patients, the majority of whom had chronic HCV infection. However, the levels did not correlate with any of the conventional staging systems or standard clinical parameters, and in aggregate the data suggest that miR-21 may aid in the diagnosis of HCC but is not suitable for screening.

While the Tomimaru study is unlikely to have a major impact on understanding HCC disease pathogenesis or improving diagnosis, it introduces the broader issue of microRNA biology, and the emerging roles of circulating miRNAs in malignant and benign liver diseases. The astonishing growth in our understanding of miRNAs since the Nobel prize-winning work of Fire and Mello in 1998 [4] continues to yield enormous scientific and clinical dividends, including a substantial refinement of our understanding of cancer pathogenesis [5], as well as prospects for new therapies [6]. MicroRNAs are endogenous non-coding RNAs whose highly regulated processing yields cytoplasmic RNA fragments (20–22 nt) that bind to specific seed sites in a range of target RNAs, thereby altering RNA stability, and/or translational activity. MicroRNAs in cancer may be up- or down-regulated and thereby may enhance tumor growth by antagonizing tumor suppressors or increasing the expression of growth-promoting genes [7]. They are part of a rapidly growing family of non-coding regulatory RNA molecules [8] that now includes ceRNAs (competing endogenous), lincRNA (large intervening non-coding), snoRNAs (small nucleolar), and PiwiRNA (P-element induced wimpy testis). Clearly, the field has come a long way from the simple *central dogma of biology*, which was originally defined as "DNA to RNA to Protein".

While changes in individual miRNAs in HCC have been extensively described, altered expression patterns of miRNA clusters are providing a more comprehensive picture of the their role in the disease, and have facilitated the identification of new therapeutic targets [9–11]. For example, miR-517a recently emerged from such a global analysis, based on its elevated expression in an aggressive subtype of human disease, and its contribution to tumorigenesis in an animal model [9].

Coming on the heels of studies exploring miRNAs in pathogenesis have been efforts to detect miRNAs in serum or plasma of patients with a range of cancers as potential biomarkers [12], as in the Tomimaru study in this issue of the *Journal*. The miRNAs detectable in serum are remarkably stable to a variety of physical conditions including boiling, freeze–thaw, and extreme pH, which has led to the discovery that these molecules can be sequestered within small membrane vesicles (50–90 nm) called exosomes. MicroRNAs within exosomes are not only potential disease markers, but can be biologically active at distant sites [13]. Indeed, HCC cells in culture can produce miRNA-containing exosomes that modulate intracellular signaling of neighboring cells [14]. Circulating miRNAs are not only present in malignant liver diseases, but also in chronic injury, since a recent study has demonstrated that a miRNA (miR-29a) derived from activated stellate cells is detectable in human serum, and its level decreases with advancing fibrosis [15].

Despite these exciting translational prospects, there remain more questions than answers in attempting to exploit miRNA biology in clinical medicine. Are all miRNAs that are detected in serum sequestered within exosomes? Are circulating miRNA-containing exosomes present in humans, and if so how can they be quantified? What is the normal range of serum miRNA levels and quantity of exosomes? What are the mechanisms underlying miRNA release from cancer and non-cancer cells, and how will answering this question advance our understanding of disease pathogenesis? Given their ability to modulate many human genes simultaneously, can miRNAs help us understand the difference between benign neoplasia and liver cancer? And finally, will combinations of miRNAs prove more useful as biomarkers of disease detection or staging than individual ones?

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Focus

Whereas the miRNA study investigated a host determinant of HCC, the study by Lee *et al.* from Korea in this issue of the *Journal* explored a viral determinant. This study of HBV stop codons in the HBV surface antigen gene traces its conceptual roots to the human papilloma virus (HPV) field, where some viral strains are known to be far more oncogenic than others. The question of whether hepatitis viruses have oncogenic strains, mutant forms, and sequence variants has recently become a hot research topic, stimulated in part by the discovery of mutations in the HCV *core* gene that are associated with increased HCC risk [16]. The study by Lee *et al.* provides an interesting mechanistic link between the increased risk of HCC among Asian patients with HBV genotype C and HBV mutations [17–19]. Genotype C, which is most common in Asia, has been associated with an increased risk of delayed HBeAg seroconversion, higher viral replication, and increased risk of fibrosis, cirrhosis, and HCC [17]. Among their genotype C patients, the Korean investigators have performed extensive viral sequence analysis to study the clinical significance of a premature stop at codon 182 in the S gene, which creates a truncated S protein. In 275 patients in whom HBV DNA could be amplified, 73 (26.5%) harbored variants encoding one of the two possible stop codons (UGA or UAG) at position 182 of the HBV surface antigen gene. In most cases, the virus population also included variants with the wild type gene. Variants encoding the stop codons were more common in patients with advanced liver disease (HCC and/or cirrhosis) than in patients without cirrhosis. Interestingly, these patients had lower HBV levels, which is surprising because higher levels of HBV replication are usually associated with more progressive liver disease. The authors speculate that these paradoxical effects on viral replication result from increased ER stress and an unfolded protein response (UPR) in infected cells, as well as impaired formation of virions and loss of infectivity. The authors also demonstrate that cell growth was increased in HCC lines stably expressing the stop codon variant, compared to those with the wild type sequence, which was linked to increased cell cycle progression and colony forming ability, and reduced p53, and p21 expression, proteins which block cell growth.

These findings raise tantalizing clues that merit further exploration. Although the authors imply that mutant viruses are driving liver disease progression, this cross-sectional analysis does not allow one to determine whether the mutations arose before or after the advanced liver injury. Also, the suggestion that the 182 stop variant induces more ER stress and UPR is eminently testable in cultured HCC cells, although levels of viral protein expression should be comparable to those observed *in vivo* rather based upon an over-expression system in which protein levels exceed those of the native infection. In analyzing the specific sequences of codon 182, a UGA stop codon was much more common than the UAG codon, even though both require a single mutation of the wild type codon (UGG-tryptophan). Significantly, the UGA stop codon introduces a mutation into the overlapping polymerase gene, while the UAG mutation does not. Absent some selection pressure, one would expect the UAG mutation to be more common than the UGA mutation, because a virus would typically want to preserve its polymerase gene. What selection pressure could favor a mutation in the polymerase gene? One well-known force that can drive such mutations is the use of antiviral drugs such as nucleoside reverse transcriptase inhibitors. The authors do not provide any information about the treatment history of the patients, but it raises concern that the use of antiviral drugs favors the creation of viruses that could interfere with the cell's ability to process proteins.

The mechanisms leading to increased cell growth are equally intriguing but obscure. The link to altered p53 is reminiscent of many studies of the HBV X protein [20], which initially were plagued by concerns about whether levels of X protein expression in cell culture models were appropriate. From a molecular/epidemiologic perspective, it would be interesting to know whether the entire risk of increased HCC associated with genotype C can be attributed to the subset of patients with this mutation, or whether other features of the genotype are also permissive for HCC development. In particular, can the increased disease activity and advanced fibrosis associated with this genotype confer an independent risk of HCC? Also, it would be interesting to know where the polymerase gene mutations map relative to the various domains of this protein.

Finally, the stop codon mutations are associated with low viral load but they are not associated with loss of HBeAg expression, which is atypical. If the association between these mutations and adverse clinical outcomes is confirmed in future studies, providers will need to be vigilant for patients with low HBV viral load who are HBeAg positive. These patients may harbor stop codons that confer an increased risk of cirrhosis and HCC.

Together, these two studies pave more inroads in unraveling the mysteries of HCC, and further highlight the disease's complexity.

Conflict of interest

The author declared that he does not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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